Nitroso-redox balance in control of coronary vasomotor tone


Experimental Cardiology, Thoraxcenter, Cardiovascular Research Institute COEUR, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Submitted 20 April 2011; accepted in final form 31 January 2012

Taverne YJ, de Beer VJ, Hoogteijling BA, Juni RP, Moens AL, Duncker DJ, Merkus D. Nitroso-redox balance in control of coronary vasomotor tone. J Appl Physiol 112: 1644–1652, 2012. First published February 23, 2012; doi:10.1152/japplphysiol.00479.2012.—Reactive oxygen species (ROS) are essential in vascular homeostasis but may contribute to vascular dysfunction when excessively produced. Superoxide anion (O$_2^-$) can directly affect vascular tone by reacting with K$^+$ channels and indirectly by reacting with nitric oxide (NO), thereby scavenging NO and causing nitroso-redox imbalance. After myocardial infarction (MI), oxidative stress increases, favoring the imbalance and resulting in coronary vasoconstriction. Consequently, we hypothesized that ROS scavenging results in coronary vasodilatation, particularly after MI, and is enhanced after inhibition of NO production. Chronically instrumented swine were studied at rest and during exercise before and after scavenging of ROS with N-(2-mercaptopyrrolinyl)-glycine (MPG, 20 mg/kg iv) in the presence or absence of prior inhibition of endothelial NO synthase (eNOS) with N$^\omega$-nitro-l-arginine (l-NNa, 20 mg/kg iv). In normal swine, MPG resulted in coronary vasodilatation as evidenced by an increased coronary venous O$_2$ tension, and trends toward increased coronary venous O$_2$ saturation and decreased myocardial O$_2$ extraction. These effects were not altered by prior inhibition of eNOS. In MI swine, MPG showed a significant vasodilator effect, which surprisingly was abolished by prior inhibition of eNOS. Moreover, eNOS dimer/monomer ratio was decreased after MI, reflecting eNOS uncoupling. In conclusion, ROS exert a small coronary vasoconstrictor influence in normal swine, which does not involve scavenging of NO. This vasoconstrictor influence of ROS is slightly enhanced after MI. Since inhibition of eNOS abolished rather than augmented the vasoconstrictor influence of ROS in swine with MI, while eNOS dimer/monomer ratio was decreased, our data imply that uncoupled eNOS may be a significant source of O$_2^-$ after MI.

myocardial infarction; swine; reactive oxygen species; superoxide; coronary blood flow; myocardial oxygen balance; exercise

NITRIC OXIDE (NO) and superoxide (O$_2^-$) are key players in cellular nitroso-redox balance and are required for normal vascular homeostasis. The importance of the nitroso-redox balance in the cardiovascular system is underlined by studies that show that oxidative stress, i.e., a disturbance of the nitroso-redox balance, contributes to the pathogenesis of diabetes, hypertension, and atherosclerosis (1, 16, 38, 49, 50, 66). Although several studies have shown that oxidative stress is increased after a myocardial infarction (MI), even in the remote myocardium (7, 9), and that the increased oxidative stress contributes to endothelial dysfunction in isolated large coronary arteries (6), the influence of the increased oxidative stress on the coronary microvasculature in the remote myocardium after MI in vivo has not been investigated to date.

Under normal physiological conditions O$_2^-$ is enzymatically produced by a variety of oxidases, including xanthine oxidase and NADPH oxidase and as a by-product of oxidative phosphorylation in the mitochondria, which is presumed to be the major source of O$_2^-$ production (18, 67). O$_2^-$ can affect vascular function either directly, by reducing the opening probability of K$^+$ channels, or indirectly, by quenching NO and forming ONOO$^-$, both leading to vasoconstriction (60). To prevent the deleterious actions of high concentrations of O$_2^-$, its concentration is tightly controlled and kept in the picomolar range by superoxide dismutase (SOD) thereby creating H$_2$O$_2$, a membrane-permeable vasodilator (37, 41) that has been suggested to be the factor that couples myocardial metabolism to coronary vasomotor tone (56).

After MI, O$_2^-$ is excessively produced, resulting in oxidative stress even in the remote, noninfarcted myocardium (7, 9), which may result in enhanced coronary vasoconstriction. Given the role of the mitochondrial respiratory chain as a major source of O$_2^-$, oxidative stress is likely to increase during exercise. An increased O$_2^-$-mediated vasoconstriction may therefore directly contribute to the relative hypoperfusion of the remote noninfarcted myocardium that is particularly observed during exercise (29). Thus we hypothesize that the vasoconstrictor effect of O$_2^-$ in the remote, noninfarcted myocardium is enhanced after MI, especially during exercise. To test this hypothesis, we investigated the effects of N-(2-mercaptopyrrolinyl)-glycine (MPG), a synthetic aminothiol antioxidant that acts primarily, although perhaps not exclusively (2, 59), through scavenging of O$_2^-$ (16), in chronically instrumented swine with and without a recent MI.

In addition to its direct coronary vasoconstrictor effect, oxidative stress may result in a shift in the nitroso-redox balance toward the formation of peroxynitrite (ONOO$^-$), thereby reducing NO bioavailability, and counteracting the physiological NO-mediated quiescent and dilated state of blood vessels (64). Moreover, O$_2^-$ can lead to uncoupling of endothelial NO synthase (eNOS), through oxidation of its cofactor tetrahydrobiopterin (BH$_4$), thereby causing a shift from NO to O$_2^-$ production (14, 31) and further aggravating microvascular dysfunction. We therefore also investigated whether eNOS uncoupling occurred in the remote myocardium after MI, and if the contribution of eNOS-dependent O$_2^-$ was altered after MI.

METHODS

Animals

Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996), and with prior
Surgery

Twenty-six swine were sedated (20 mg/kg ketamine and 1 mg/kg midazolam im), anesthetized (thiopental sodium 15 mg/kg iv), intubated, and ventilated with a mixture of O₂ and N₂ (1:2) to which, if necessary, 0.2–1.0% (vol/vol) isoflurane was added (21, 42). Anesthesia was maintained with midazolam (2 mg/kg + 1 mg·kg⁻¹·h⁻¹ iv) and fentanyl (10 μg·kg⁻¹·h⁻¹ iv). Under sterile conditions, the chest was opened via the fourth left intercostal space, and a fluid-filled polyvinylchloride catheter was inserted into the aortic arch for aortic blood pressure measurement (Combibra pressure transducers, Braun) and blood sampling. A Transit-time flow probe (Transonic Systems) was positioned around the ascending aorta via the apex. Polyvinylchloride catheters were inserted into the left ventricle (LV) to calibrate the Konigsberg transducer LV pressure signal, into the left atrium to measure pressure, and into the pulmonary artery to administer drugs. A small angiocatheter was inserted into the anterior interventricular vein for coronary venous blood sampling. Finally, a transit-time flow probe (Transonic Systems) was placed around the left anterior descending coronary artery (42). In all swine the proximal part of the left coronary circumflex artery (LCx) was exposed, but only in 14 animals was the LCx permanently occluded with a silk suture to produce a MI (29). Catheters were tunneled to the back, and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamicin iv) for 5 days (21, 42). Two MI swine died overnight, most likely due to ventricular fibrillation.

Experimental Protocols

Studies were performed 1–3 wk after surgery with animals resting and exercising on a motor-driven treadmill up to 85–90% of maximal heart rate. Two protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma.

Effect of scavenging of ROS. With swine lying quietly on the treadmill, resting hemodynamic measurements consisting of heart rate, LV pressure, first derivative of LV pressure (dP/dt), mean aortic pressure, left atrial pressure, aortic and coronary blood flow (CBF) were obtained and arterial and coronary venous blood samples collected in 8 normal and 11 MI swine. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, swine were subjected to a four-stage exercise protocol (1–4 km/h) while hemodynamic variables were continuously recorded and blood samples collected during the last 60 s of each 3 min exercise stage at a time when hemodynamics had reached a steady state. After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min after which animals received the ROS scavenger MPG [1 mg·kg⁻¹·min⁻¹, continuous infusion (34, 44, 45, 48, 62)] and the exercise protocol was repeated.

We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise (20, 21).

Effect of ROS scavenging after NO blockade. Ninety minutes after 11 normal and 8 MI swine had undergone a control exercise trial, animals received NO synthase inhibitor Nω-nitro-L-arginine (L-NNA, 20 mg/kg iv) (20) and underwent a second exercise trial. Ninety minutes later, MPG (1 mg·kg⁻¹·min⁻¹, continuous infusion) was given to the animals and they underwent a third exercise trial. Because L-NNA has a long-lasting effect (mean arterial pressure, 123 ± 4 mmHg after administration of L-NNA before the second exercise trial and 121 ± 4 mmHg after administration of MPG prior to the third exercise trial), no additional t- NNA was administered before the third exercise protocol.

Blood gas measurements. Blood samples were kept in iced syringes until the conclusion of each exercise trial. Measurements of PO₂ (mmHg), Pco₂ (mmHg), and pH were then immediately performed with a blood-gas analyzer (model 600, Acid-Base Laboratory, Radiometer, Copenhagen, Denmark). Hemoglobin (Hb; g/100 ml) and Hb O₂ saturation (S₀₂) were measured with a hemoximeter (OSM3, Radiometer). Myocardial O₂ delivery (MDO₂ = CBF × arterial O₂ content), myocardial O₂ consumption [MVO₂ = CBF × (arterial O₂ content – coronary venous O₂ content)], and myocardial O₂ extraction (MEO₂ = MVO₂/MDO₂) were computed using the blood-gas values and CBF (21).

It is noteworthy that pigs have a negligible native collateral circulation, so that an acute occlusion of the LCx (without reperfusion) results in a transmural MI of the lateral wall encompassing 20–25% of the LV (29, 57). Even though 1–3 wk after LCx occlusion some “collateral” flow to the fibrotic infarct zone is present (~20% of flow to the normal myocardium (68)), it is important to note that the anterior interventricular vein selectively drains the LAD perfusion territory (8), so that changes in O₂ balance in the infarcted area will not be reflected in the coronary venous O₂ content in the anterior interventricular vein.

eNOS Uncoupling

A separate group of swine (n = 12) was used for determination of eNOS uncoupling. Initial surgery was performed as described above, but no catheters were implanted. The LCx coronary artery was dissected free in all 12 swine, and ligated in 6 swine to induce MI. All 12 swine were euthanized 3 wk after induction of MI or sham operation. One MI pig was excluded from analysis due to a very small infarct size (<10% of LV).

Subendocardial tissue from the remote, noninfarcted anterior free wall was used. Myocardium was homogenized for 10 s in lysis buffer of the following composition: 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% SDS; 0.5% deoxycholate; 1% NP-40; protease inhibitors; 1 mM PMSF. Samples (60 μg of protein) were separated on a gel containing 0.375 M Tris, pH 8.8. Low-temperature SDS-PAGE was performed for detection of eNOS monomer and dimer (46). The protein samples were subjected to SDS-PAGE with 7% self-made SDS-Tris gels run overnight. Gels and buffers were equilibrated at 4°C before electrophoresis, and the buffer tank was placed in an ice bath during electrophoresis to maintain the low temperature. Subsequent to SDS-PAGE, the proteins were transferred for 3 h to nitrocellulose membranes. The blots were then probed as routine Western blot with primary NOS3 antibody (1:5,000, Santa Cruz Biotechnology, Heidelberg, Germany) and secondary rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; 1:1,000, Santa Cruz Biotechnology, Heidelberg, Germany), and detected by enhanced chemiluminescence substrate (Perkin Elmer) with LAS 3000 CCD camera (Fujifilm). The images were then analyzed with ImageJ (NIH). Data are presented as arbitrary units, which are a combination of band size and intensity.

Data Analysis

Hemodynamic data were digitally recorded and analyzed off-line. Effects of free radical scavenging (MPG) and eNOS inhibition (t- NNA) and MI on systemic hemodynamics were assessed with ANOVA for repeated measures followed by post hoc tests (Scheffe) where appropriate. Statistical comparison of individual data points in the table between normal and MI swine was performed using an unpaired t-test. To test for the effects of MI and drug treatment (MPG and/or t-NNA) on the relation between MVO₂ and coronary venous O₂ tension (cVPO₂), coronary venous O₂ saturation (cVSO₂), or MEO₂, regression analysis was performed using MI, drug treatment, and

J Appl Physiol • doi:10.1152/japplphysiol.00479.2011 • www.jappl.org
MVO₂ as well as their interaction as independent variables and assigning a dummy variable to each animal. Band densities of the Western blots were compared between normal and MI swine using an unpaired t-test. Statistical significance was accepted at $P \leq 0.05$ (2 tailed). Data are presented as means $\pm$ SE.

RESULTS

**Systemic Hemodynamics and LV Function**

Despite the loss of viable myocardial tissue, encompassing 20–25% of the LV (29, 57), the LV weight-to-body weight ratio in MI swine (3.5 $\pm$ 0.1 g/kg) tended to be higher than in normal swine (3.3 $\pm$ 0.2 g/kg), reflecting hypertrophy of surviving myocardium. MI resulted in LV dysfunction, as evidenced by a lower stroke volume, LV systolic pressure, and LV $dP/dt_{\text{max}}$, and a twofold higher mean left atrial pressure (all $P < 0.05$ by ANOVA). Exercise resulted in blunted increments of stroke volume and LV $dP/dt_{\text{max}}$ in MI compared with normal swine, while the increase in heart rate was maintained (Fig. 1, Table 1).

Scavenging of ROS through administration of MPG had little effect on LV function in either normal or MI swine as LV systolic pressure (Fig. 1), left atrial pressure (Table 1), and the relation between heart rate and $dP/dt_{\text{max}}$ were unaffected by administration of MPG (Fig. 1). Mean arterial pressure decreased slightly in both normal and MI swine. The decrease in mean arterial pressure was accompanied by an increase in heart rate in normal swine, while stroke volume was slightly reduced (Table 1), resulting in an unaltered cardiac output. In swine with MI, both heart rate and stroke volume remained unaffected by administration of MPG. Moreover, radical scavenging had no significant effect on peripheral vascular tone as systemic vascular conductance (SVC) did not change significantly after administration of MPG (Fig. 1).

---

**Fig. 1.** Changes in cardiac function and systemic hemodynamics in response to N-(2-mercaptoproprionyl)-glycine (MPG) in normal swine (N) and swine with myocardial infarction (MI) in the absence and presence of endothelial nitric oxide synthase (eNOS) inhibition with N$^\text{ω}$-nitro-L-arginine (L-NNA). $dP/dt_{\text{max}}$, maximum of first derivative of left ventricular pressure as an index of contractility; LVSP, left ventricular systolic pressure; MAP, mean arterial pressure; SVC, systemic vascular conductance; $0_\text{L}$ is rest, lying; $0_\text{S}$ is rest, standing. Data are means $\pm$ SE. *$P < 0.05$, effect of MPG. †$P < 0.05$, control relation different in normal swine vs. swine with MI.
Effect of MPG on hemodynamics in the presence and absence of NO synthase inhibition in healthy and MI swine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Behavior</th>
<th>CO, l/min</th>
<th>Exercise Level, km/h</th>
<th>LAP, mmHg</th>
<th>HR, beats/min</th>
<th>SV, ml</th>
<th>CO, l/min</th>
<th>LAP, mmHg</th>
<th>HR, beats/min</th>
<th>SV, ml</th>
<th>CO, l/min</th>
<th>LAP, mmHg</th>
<th>HR, beats/min</th>
<th>SV, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI Rest. Lying</td>
<td>179 ± 10*</td>
<td>137 ± 8</td>
<td>37 ± 3 §</td>
<td>132 ± 3</td>
<td>137 ± 9</td>
<td>33 ± 2</td>
<td>136 ± 10</td>
<td>167 ± 7</td>
<td>32 ± 2</td>
<td>31 ± 3</td>
<td>173 ± 11</td>
<td>134 ± 12</td>
<td>25 ± 4</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>MI MPG</td>
<td>192 ± 10*</td>
<td>155 ± 9</td>
<td>38 ± 3</td>
<td>153 ± 3</td>
<td>157 ± 10</td>
<td>33 ± 2</td>
<td>136 ± 10</td>
<td>185 ± 7</td>
<td>34 ± 2</td>
<td>33 ± 2</td>
<td>178 ± 7</td>
<td>134 ± 12</td>
<td>26 ± 4</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>MI l-NNA</td>
<td>214 ± 12*</td>
<td>98 ± 4</td>
<td>8.3</td>
<td>154 ± 7</td>
<td>155 ± 7</td>
<td>176 ± 7</td>
<td>198 ± 8</td>
<td>190 ± 5</td>
<td>210 ± 10</td>
<td>176 ± 7</td>
<td>173 ± 7</td>
<td>204 ± 7</td>
<td>204 ± 7</td>
<td>204 ± 7</td>
</tr>
<tr>
<td>MI l-NNA/MPG</td>
<td>229 ± 11*</td>
<td>115 ± 7</td>
<td>154 ± 7</td>
<td>121 ± 7</td>
<td>154 ± 7</td>
<td>187 ± 8</td>
<td>190 ± 5</td>
<td>210 ± 10</td>
<td>204 ± 7</td>
<td>173 ± 7</td>
<td>204 ± 7</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>MI MPG +</td>
<td>244 ± 7</td>
<td>136 ± 9</td>
<td>8.3</td>
<td>154 ± 7</td>
<td>155 ± 7</td>
<td>176 ± 7</td>
<td>198 ± 8</td>
<td>190 ± 5</td>
<td>210 ± 10</td>
<td>176 ± 7</td>
<td>173 ± 7</td>
<td>204 ± 7</td>
<td>204 ± 7</td>
<td>204 ± 7</td>
</tr>
<tr>
<td>MI l-NNA +</td>
<td>201 ± 9</td>
<td>130 ± 12</td>
<td>154 ± 7</td>
<td>121 ± 7</td>
<td>154 ± 7</td>
<td>187 ± 8</td>
<td>190 ± 5</td>
<td>210 ± 10</td>
<td>204 ± 7</td>
<td>173 ± 7</td>
<td>204 ± 7</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>MI l-NNA/MPG +</td>
<td>229 ± 11*</td>
<td>134 ± 12</td>
<td>167 ± 7</td>
<td>181 ± 8</td>
<td>186 ± 7</td>
<td>24 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
</tr>
</tbody>
</table>

Data are means ± SE. Con, control; MPG, N-(2-mercaptoproprionyl)-glycine; HR, heart rate; SV, stroke volume; CO, cardiac output; LAP, left atrial pressure, MI, myocardial infarction. *P < 0.05 vs. rest; †P < 0.05, effect of MPG; ‡P < 0.05, effect of l-NNA; §P < 0.05 vs. normal swine.

eNOS blockade with l-NNA resulted in peripheral vasoconstriction as evidenced by a decrease in SVC and an increase in mean arterial pressure (Fig. 1). The increase in mean arterial pressure was accompanied by increases in LV systolic pressure as well as left atrial pressure, and was accompanied by a probably baroreflex-mediated decrease in heart rate and cardiac output, as stroke volume was not altered (Table 1). The effects of eNOS inhibition were similar in normal swine and swine with MI.

In the presence of l-NNA, ROS scavenging with MPG resulted in a small but significant increase in SVC and a decrease in mean arterial pressure, reflecting mild systemic vasodilation. The decrease in mean arterial pressure resulted in a probably baroreceptor reflex-mediated increase in heart rate and cardiac output. The decreases in LV systolic pressure and left atrial pressure following MPG administration in the presence of l-NNA most likely occurred in parallel to the decrease in mean arterial blood pressure, as neither stroke volume (Table 1) nor the relation between heart rate and LV dP/dtmax changed (Fig. 1). The systemic hemodynamic effects of MPG after eNOS inhibition were similar in normal swine and swine with MI.

Coronary Circulation

In accordance with previous studies from our laboratory (20, 30, 43), coronary blood flow and myocardial O2 delivery...
increased commensurate with the exercise-induced increase in myocardial O₂ consumption, so that cvSO₂ and cvPO₂ remained constant (Fig. 2). After MI, the increase in coronary blood flow to the remote, noninfarcted myocardium was slightly less than the increase in myocardial O₂ consumption, forcing the heart to increase its O₂ extraction as reflected by a slight decrease in particularly cvSO₂ with increasing exercise intensity, while cvPO₂ was less affected (Fig. 2).

In normal swine, MPG resulted in coronary vasodilation as evidenced by a significant increase in cvPO₂ and a tendency toward an increase in cvSO₂ (P = 0.13) and decreased MEO₂ (P = 0.15). The effects of MPG were similar at rest and during exercise (Fig. 2). In the remote coronary vasculature of swine with MI, the vasodilator effect of MPG was significantly increased, as the shifts in cvSO₂ were significantly larger compared with normal swine (Fig. 2), indicating increased oxidative stress in the coronary vasculature.

In accordance with previous studies from our laboratory (20, 30, 43), inhibition of eNOS with L-NNA resulted in coronary vasoconstriction in both normal swine and swine with MI, as evidenced by a decrease in cvSO₂ and cvPO₂ and an increase in MEO₂ (Fig. 2). Subsequent administration of MPG resulted in small but significant vasodilation in normal swine (Fig. 2). However, the effect of MPG after L-NNA was not different from its effect under control conditions, suggesting that ROS exert a direct effect on the coronary vasculature that is not mediated through scavenging of NO. Surprisingly, in swine with MI, MPG had no effect on cvSO₂ or cvPO₂ in the presence of L-NNA (Fig. 2). Thus the vasodilator effect of MPG was abolished by prior eNOS inhibition, suggesting that, in the remote myocardium of swine with MI, eNOS contributes to formation of ROS, most likely O₂⁻⁻, which is in accordance with our recent observation that DHE staining was increased in the remote myocardium after MI (9).

eNOS Uncoupling

Total eNOS as well as the eNOS dimer-monomer ratio were decreased in tissue from swine with MI compared with normal swine (Fig. 3), indicating that eNOS expression was decreased whereas eNOS uncoupling was increased in swine with MI.

DISCUSSION

The major findings of the present study are that 1) ROS cause coronary vasoconstriction in normal swine that is not mediated through altered bioavailability of NO; 2) the vasoconstrictor influence of ROS is enhanced in the remote myocardium after MI; 3) inhibition of eNOS reduced rather than enhanced the vasoconstrictor influence of ROS after MI, and 4) eNOS dimer-monomer ratio was decreased in myocardial tissue from swine with MI, suggesting that (uncoupled) eNOS is a significant source of O₂⁻⁻. Implications of these findings will be discussed below.

Methodological Considerations

Choice of MPG. We chose to use MPG as a ROS scavenger as MPG is not highly radical specific and scavenges different types of ROS, including O₂⁻⁻, ONOO⁻ and OH⁻ (2, 59). Although radiolabeling showed that MPG primarily accumulates in the mitochondria (13), it is highly diffusible and has been shown to scavenge radicals both in the intracellular and extracellular compartment (10). Thus MPG is able to access most sources of free radical production (35, 52), which allowed us to assess the effects of changes in the cellular nitroso-redox balance on coronary vascular tone independent of the source of free radicals. Our observation that administration of MPG resulted in vasodilation suggests that MPG acts through scavenging of a vasoconstrictor ROS, most likely O₂⁻⁻. In the
remainder of the discussion we will, therefore, focus on the role of $O_2^-$, although a contribution of other vasoconstrictor ROS like ONOO$^-$ and OH cannot be excluded.

**Myocardial $O_2$ balance as an index of coronary vascular tone.** Intravenous administration of blockers of vasoactive systems results in changes in systemic hemodynamics that not only affect myocardial oxygen demand but also affect coronary perfusion pressure and perfusion time, and may therefore result in autoregulatory and metabolic changes in resistance vessel tone (29, 65). Changes in CBF or coronary vascular resistance following blockade of a vasoactive system therefore reflect not just the response to the blocker that was administered. Under basal resting conditions, the heart is characterized by a high level (80%) of $O_2$ extraction (21, 22). Accordingly, the ability of the coronary resistance vessels to dilate in response to increments in myocardial $O_2$ demand is extremely important to maintain an adequate $O_2$ supply to the myocardium. A sensitive way to study alterations in coronary vascular tone in relation to myocardial metabolism, independent of changes in systemic hemodynamics, is the relationship between coronary venous $O_2$ levels and myocardial $O_2$ consumption (29, 65). Thus an increased coronary resistance vessel tone will reduce coronary blood flow, and hence myocardial $O_2$ delivery, at a given level of myocardial $O_2$ consumption, forcing the myocardium to increase its $O_2$ extraction in order to meet myocardial $O_2$ demand, thus resulting in a lower coronary venous $O_2$ level. Conversely, a decrease in resistance vessel tone increases myocardial $O_2$ delivery at a given level of myocardial $O_2$ consumption, resulting in an increased coronary venous $O_2$ level. Coronary venous $O_2$ levels thus represent an index of myocardial tissue oxygenation (i.e., the balance between $O_2$ supply and $O_2$ demand) that is determined by coronary resistance vessel tone. Accordingly, the relations between $MV_{O_2}$ and $cvSO_2$, and $cvPO_2$ were used for interpretation of changes in coronary resistance vessel tone.

**Effects of Nitroso-Redox Balance on Cardiac Function**

Oxidative modification of calcium transporters (75) as well as contractile proteins (19) has been shown to contribute to cardiac dysfunction. In the present study, we found a slight decrease in stroke volume, but no further evidence for altered cardiac function, after scavenging free radicals with MPG in normal swine, suggesting that free radicals produced in the normal heart are effectively scavenged by normal antioxidant mechanisms, and therefore do not affect cardiac function.

After MI, cardiac function is hampered due to loss of functional myocardial cells, leading to impaired ventricular contraction, as evidenced by decreases in LV systolic pressure, LV dP/dmax, and stroke volume, which results in elevated LV filling pressures in an attempt to maintain function. A recent study from our laboratory (9) showed that DHE staining of the remote myocardium of swine with MI is increased compared with normal animals, indicating that $O_2^-$ generation is increased. Thus oxidative modification of myofilaments may contribute to the observed LV dysfunction after myocardial infarction. Indeed, cardiac myocytes, isolated from the remote myocardium, displayed both systolic (reduced maximal force) and diastolic (increased calcium sensitivity) dysfunction (69). Nevertheless, administration of MPG did not alter global cardiac function in swine with MI, indicating that an acute reduction in oxidative stress with MPG did not immediately improve LV function.

**Effects of Nitroso-Redox Balance on the Vasculature**

**Healthy swine.** The effect of ROS is determined by their rate and location of production as well as by their rate and route of degradation. $O_2^-$ generation occurs in all layers of the vascular wall (64). Because it is highly reactive, non- membrane permeable and because antioxidant defense mechanisms are abundantly present, the actions of $O_2^-$ will be restricted to the subcellular compartment in the proximity of its source of generation (24, 51, 55, 63). There are several ways in which $O_2^-$ may alter vascular tone. Thus $O_2^-$ can react with NO to form ONOO$^-$, thereby limiting NO bioavailability and decreasing NO-mediated vasodilation (23). Moreover, $O_2^-$ can oxidatively alter various $K^+$ channels in vascular smooth muscle cells (28, 40), thereby depolarizing the membrane potential and resulting in vasoconstriction. In addition, $O_2^-$

---

*Fig. 3. Changes in eNOS expression (dimer + monomer) and eNOS uncoupling (dimer/monomer ratio) in normal swine and swine with MI. Data are means ± SE. *P < 0.05, effect of MI.*
can react with the sarco(endo)plasmic reticulum Ca\(^{2+}\) (SERCA) pump in pig coronary artery smooth muscle cells, while plasma membrane Ca\(^{2+}\) pumps are relatively resistant to oxidative modification (25, 26). However, SERCA inhibition by ONOO\(^{-}\) depletes the SR from Ca\(^{2+}\) and is therefore more likely to induce vasodilation (27, 71).

In healthy subjects, O\(_2\)\(^{-}\) levels are tightly controlled by superoxide dismutase (SOD) (32, 36, 39, 61). SOD catalyzes the reaction of O\(_2\)\(^{-}\) into H\(_2\)O\(_2\) and O\(_2\). The abundance of SOD in healthy blood vessels limits the amount of ONOO\(^{-}\) formed under normal conditions although the reaction between NO and O\(_2\)\(^{-}\) to form ONOO\(^{-}\) is 3–4 times faster than the reduction of O\(_2\)\(^{-}\) by SOD (5). H\(_2\)O\(_2\), a potent vasodilator (37, 41, 54, 56) that is formed by SOD, is more stable and diffusible than O\(_2\)\(^{-}\) and is, therefore, under normal physiological conditions likely to affect vascular tone. Consequently, the net effect of ROS scavenging with MPG on vascular tone depends on the relative quantities of O\(_2\)\(^{-}\) and H\(_2\)O\(_2\).

ROS scavenging with MPG had no significant effect on vascular tone in the systemic circulation, suggesting that either the amount of free radicals produced is very low or that the vasoconstrictor influence of O\(_2\)\(^{-}\) is balanced by a similar vasodilator influence of H\(_2\)O\(_2\). After inhibition of eNOS, however, administration of MPG did result in slight but statistically significant systemic vasodilation. Hence, our data indicate that minimal scavenging of O\(_2\)\(^{-}\) by NO does occur, which limits the direct effect of O\(_2\)\(^{-}\) on the systemic vasculature.

In the coronary circulation, however, free radical scavenging with MPG did have a small but significant vasodilator effect both at rest and during exercise, indicating that the vasoconstrictor effect of O\(_2\)\(^{-}\) exceeds the vasodilator effect of H\(_2\)O\(_2\), resulting in a net vasoconstrictor effect of ROS on the coronary vasculature. We did not investigate the source of the free radicals, but the observation that inhibition of NADPH-oxidase did not affect vascular tone in awake dogs (74) suggests that NADPH-oxidase is not the source of O\(_2\)\(^{-}\) in the coronary vasculature of healthy animals. Recent studies in rats imply that the respiratory chain of the mitochondria in the cardiac myocytes is the main source of O\(_2\)\(^{-}\) (56). O\(_2\)\(^{-}\) derived from the mitochondria is converted by SOD to the vasodilator H\(_2\)O\(_2\), which is membrane permeable, diffuses to the microvasculature, and is responsible for the coupling of increased myocardial metabolism to vasodilation of the coronary microvessels (56, 73). During exercise, myocardial O\(_2\) consumption doubles and even moderate exercise may increase mitochondrial free radical production, possibly beyond endogenous antioxidant defenses, resulting in oxidative stress (17, 53, 58). However, administration of a SOD mimetic had no effect on coronary vasomotor tone in awake dogs either at rest or during exercise (12), indicating that endogenous SOD is capable of converting the majority of O\(_2\)\(^{-}\). These findings are in accordance with the findings in the present study that the effect of MPG was not increased during exercise. The apparent discrepancy between studies implying a prominent vasodilator role for endogenous O\(_2\)\(^{-}\)-derived H\(_2\)O\(_2\) and the present study that shows that scavenging O\(_2\)\(^{-}\) results in coronary vasodilation (and consequently that O\(_2\)\(^{-}\) exerts a vasoconstrictor influence) may be due to different locations where MPG and SOD act with respect to the possible sources of O\(_2\)\(^{-}\) in the vasculature.

Interestingly, eNOS inhibition appears to have very little effect on subsequent ROS scavenging with MPG (present study) or on the vasoconstrictor effect of catalase on canine coronary arterioles in vivo (73), suggesting that O\(_2\)\(^{-}\) exerts a direct vasoconstrictor effect on the coronary vasculature rather than an effect that is mediated through altered bioavailability of NO.

Altogether, the data in the present study suggest that O\(_2\)\(^{-}\) has a small direct vasoconstrictor effect on the coronary vasculature that is not mediated through altering bioavailability of NO and that is not altered during exercise.

Enhanced contribution of ROS to vascular tone after MI. Several studies have indicated that MI may result in oxidative stress in the systemic vasculature (3, 32, 33). The increased systemic oxidative stress usually occurs within days to weeks after MI (3, 33) and may be secondary to neurohumoral activation and subsequent activation of NADPH oxidase (4). In the present study, the effects of MPG in the systemic vasculature were similar in normal and MI swine, indicating that MI did not result in a generalized increase in oxidative stress.

In contrast to our findings in the systemic vasculature, the coronary vasodilator effects of MPG (present study), as well as the DHE staining of remote myocardium (9), were increased in swine with MI compared with normal swine, indicating an increased ROS production even in the microvasculature supplying the remote, noninfarcted myocardium. These data are in accordance with findings that O\(_2\)\(^{-}\) production is increased in the remote coronary arteries of rats with MI (6) as well as in monocytes/macrophages within the intima, media, and adventitia in vessels with coronary artery disease (11). NADPH oxidase has been suggested as a potential source of O\(_2\)\(^{-}\) in the remote myocardium after MI (7). Moreover, in a dog model of LV failure as a result of chronic rapid pacing (74), inhibition of NADPH oxidase with apocynin resulted in significant coronary vasodilation, suggesting that NADPH oxidase was the source of O\(_2\)\(^{-}\) in these animals with LV dysfunction.

An alternative cause of the increased O\(_2\)\(^{-}\) in the coronary vasculature may be its decreased scavenging by NO. eNOS expression was decreased in remote myocardium of MI swine in the present study, although such decrease was not found in a recent study from our laboratory in a different group of MI swine (15). An explanation for these discordant results is not readily found, but the decrease in eNOS seemed to be related to infarct size in the present study as in one swine, with a very small MI (<10% of the left ventricle), that was excluded from the analysis, eNOS expression was found to be similar as in normal swine. Moreover, since we did not use a loading control in the present study, we cannot fully exclude that subtle differences in protein loading may have influenced our results. Importantly, eNOS uncoupling, as measured by the eNOS dimer/monomer ratio (being independent of protein loading), was increased in swine with MI. eNOS uncoupling by oxidation of its cofactor BH\(_4\) and/or substrate depletion results in generation of O\(_2\)\(^{-}\) rather than NO by eNOS (47, 70, 72). Consistent with a role of eNOS in generation of O\(_2\)\(^{-}\), the vasodilator effect of MPG in swine with MI disappeared after L-NNA. Nevertheless, L-NNA resulted in significant coronary vasoconstriction at rest and during exercise, indicating that despite the decreased eNOS expression and the increased eNOS uncoupling, eNOS-mediated NO production still exerted a net coronary vasodilator effect. In accordance with these findings, agonists were still capable of inducing NO-dependent coronary vasodilation, albeit that this vasodilation was im-
paired in the remote coronary vasculature after MI (30), which is consistent with the reduced eNOS expression in the present study.

Altogether, the data in the present study suggest that uncoupled eNOS is the most important source of O$_2^-$ in the remote coronary vasculature after MI. It is however possible that O$_2^-$ production by NADPH oxidase provides the initial O$_2^-$, which acts to uncouple eNOS, thereby initiating a process that results in more O$_2^-$ production.

Conclusions

Our results suggest that ROS, and most likely O$_2^-$, contribute to coronary vasoconstriction in normal swine that is not mediated through altered bioavailability of NO. The contribution of ROS to vascular tone is enhanced after MI. The observation in MI swine that eNOS inhibition reduced rather than augmented the coronary vasoconstrictor influence of ROS, while the eNOS dimer/monomer ratio was decreased, suggests that after MI, uncoupled eNOS acts as a significant source of O$_2^-$.

GRANTS

This work was supported by grants from the Netherlands Heart Foundation (2000T042 to D. Merkus and V. J. de Beer and 2009B023 to A. L. Moens) and NWO (VIDI to A. L. Moens).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


60. Touyz RM, Schiffrin EL. Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. Hypertension 34: 976–982, 1999.


